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13. SUPPLEMENTARY NOTES

14. ABSTRACT The aim of this project is to dissect the role of Tbx2 in prostate cancer progression. In this period of the study, we found that blocking endogenous Tbx2 expression in PC3 cells by using a dominant negative construct (PC3-Tbx2 DN) reduced the osteolytic burden of these cells when placed in the bone microenvironment as compared to control PC3 cells. This finding suggests that Tbx2 plays a role in the growth of prostate cancer cells in the bone. Further, to study the role of Tbx2 in bone metastasis, we created a mouse model overexpressing hepsin and myc in the mouse prostate. This bigenic model develops adenocarcinoma earlier than the myc alone mouse and at higher ages develops higher grade adenocarcinoma.

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Statement of Work:

Title: Investigating the role of Tbx2 in the inhibition of senescence in prostate cancer.

Task 1: Characterize androgen regulation of Tbx2.

a) Identify the binding sites for androgen receptor on Tbx2 promoter. (Months 1-5)
b) Determine if the androgen receptor binds alone or in conjunction with Foxa1 to regulate Tbx2 expression. (Months 6-11)

Task 2: Determine the effect of signaling between androgen receptor and BMP2 on Tbx2 expression in the formation of prostate tumors and growth in bone microenvironment.

a) Generate stable clones of prostate cancer cells in which Tbx2 is over-expressed or down-regulated. (Months 12-16)

b) Using the derived prostate tumor cell lines that are positive or negative for Tbx2 expression, we will use different *in vivo* models (graft techniques) to delineate the contribution of Tbx2 in the progression of prostate cancer. (Months 16-24)

Task 3: Determine the role of Tbx2 signaling in resistance to pharmacological agents in human prostate cancer cells.

a) Determine the contribution of Tbx2 in resistance to chemotherapeutic agents *in vitro* using cells positive or negative for Tbx2 expression. (Months 24-28)
b) Use *in vivo* grafts to delineate the effect of modulating Tbx2 levels on the resistance to chemotherapeutic drugs. (Months 28-36)

Introduction:

Prostate cancer is the second leading cause of cancer deaths in men. Initially all prostate tumors respond to anti-androgen therapy but in almost all cases, the tumors become androgen insensitive. The majority of prostate tumors metastasize to the bone and bone metastases are the primary cause of death in prostate cancer cases. Senescence has in recent years been identified as a mechanism that can influence cancer therapy. As opposed to apoptosis or cell death, senescence can be induced by relatively low drug concentrations thus circumventing the problem of drug resistance and or drug toxicity issues in tumors. Thus identifying mechanisms by which senescence can be induced in prostate cancer cells can be a potential approach to the treatment of prostate cancer both at the primary site as well as bone metastases. Bone metastases in human prostate cancer patients are associated with osteogenic burden and are a major cause of morbidity and mortality. Several growth factors secreted by prostate cancer cells and present in the bone, including BMPs and WNTs, are known to mediate this effect. Therefore identifying factors that mediate the osteogenic effect of human prostate cancer cells in bone is relevant to therapeutic intervention. Tbx2 is a transcription factor that was first identified in a senescence screen and acts at the transcriptional level by negatively regulating p21 in human, and p19 ARF in mouse. Previously, Tbx2 has been found to be over-expressed in BRCA1 and BRCA2 associated tumors as well as melanoma and pancreatic cancer cell lines. In developmental studies, Tbx2 has been shown to form positive regulatory loops with SHH and BMP2 signaling. Interestingly, in a recent report, it was found that Tbx2 expression is increased 10-fold at the mRNA level in a highly osteoblastic metastatic bone lesion of a prostate cancer patient when compared to the highly osteolytic PC3 prostate cancer cells.

We found (in the preliminary data submitted in the original DOD grant application) that Tbx2, an anti-senescence transcription factor is over-expressed in a subset of human and mouse prostate cancer cell lines. Further we showed (in the original grant) that Tbx2 expression is regulated by androgens both in LNCaP cells and in the mouse prostate *in vivo*. Based on this preliminary data, Aim 1 proposes to study the androgen regulation of Tbx2. In Aim 2, we have proposed to study the *in vivo* effects of Tbx2 manipulation in prostate cancer cells using a tissue recombination approach to simulate the prostate microenvironment and also to study these Tbx2 manipulated

prostate cancer cells in a bone microenvironment using the technique of bone intra-tibial inoculation. Aim 3 proposes to study the role of Tbx2 in the resistance to pharmacological agents like doxorubicin in prostate cancer cells. Overall, the grant proposes to study the role of Tbx2 in prostate cancer progression.

Body:

Task 1: Characterize androgen regulation of Tbx2.

Progress on Task 1 has been described in the report of 2008.

Task 2: Determine the effect of signaling between androgen receptor and BMP2 on Tbx2 expression in the formation of prostate tumors and growth in bone microenvironment (Months 12-24):

In order to test the role played by Tbx2 in the growth of prostate cancer cells in bone microenvironment, we inoculated ($2x10^5$ cells in 10ul PBS) of either PC3-EV or PC3-Tbx2 DN cells utlizing the technique of intra-tibial inoculation in the tibia of nude mice. As a control, the contra-lateral tibia of the mouse was injected with 10 ul PBS. Intra-tibial inoculation (Fig. 1) is a technique which allows us to study the growth and effect of tumor cells on the bone microenvironment. In this technique, tumor cells are injected into the tibia of mice, the injected cells form lesions in the bone and the nature of these lesions, whether osteoblastic (bone forming) or osteolytic (bone depleting) is monitored and analyzed by weekly X-rays and the size of lesions is quantitated by using micro CT analyses.

PC3 cells when injected in the tibia are known to be osteolytic in nature. As analyzed by x-ray analysis after 3 weeks of inoculation, we found that PC3-Tbx2 DN cells were reduced in their ability to elicit an osteolytic response in the bone as compared to PC3-EV cells (Fig. 2). Also, the size of PC3-Tbx2 DN lesions was smaller as compared to PC3-EV cell lesions as seen on X-ray as well as by H&E analyses (Fig. 3) of bone tibial sections performed after fixation of harvested tibiae. We are now increasing the number of mice in each group and quantitating the size of lesions by micro CT analysis.

In order to study Tbx2 role in bone metastases, we were also interested in developing a mouse model where the metastasis originates from the mouse prostate rather then a direct graft of cells

within the tiba. Hepsin is a cell surface protease that is over-expressed in more than 90% of human prostate cancer cases. Our lab has previously developed the PB-hepsin/LPB-Tag bigenic mouse model of prostate cancer that develops bone metastases. This model is the only existing model of prostate cancer bone metastases and demonstrates that hepsin promotes primary tumors that are a mixture of adenocarcinoma and neuroendocrine lesions, and metastases that are neuroendocrine in nature. Since we are interested in studying bone metastases in prostate cancer, we wanted to study the role of hepsin in the progression of the adenocarcinoma and eventual metastasis to the bone. To address this question, we crossed the PB-hepsin mice with PB-Hi-myc transgenic mouse model of prostate adenocarcinoma. We have found that PB-hepsin/PB-Hi-myc bigenic mice develop invasive adenocarcinoma at 4.5 months. Further, histological analysis of the 12-17 month old mice revealed that the PB-hepsin/PB-Hi-myc model develops a higher grade adenocarcinoma compared with age-matched tumors expressing only PB-Hi-myc. Consistent with targeting hepsin to the prostate, the PB-hepsin/PB-Hi-myc tumors showed higher hepsin expression as compared to the age matched myc tumors. In addition, based on a previous study conducted in collaboration with our lab in which it was found that hepsin *in vitro* cleaves Laminin-332, a basement membrane component (Ref 1); we looked at Laminin-332 expression in hepsin/myc and myc alone mice. Furthermore, endogenous expression of hepsin increased in the PB-Hi-myc mice as the tumors progressed. Finally, the expression of hepsin resulted in the degradation of Laminin-332, a basement membrane component that is lost during the progression of human prostate cancer. Although we did not detect any bone metastases from the prostates in either the PB-hepsin/PB-Hi-myc or the PB-Hi-myc mice, our data suggests that hepsin and myc co-operate during the progression to high grade prostatic adenocarcinoma.

Task 3: Determine the role of Tbx2 signaling in resistance to pharmacological agents in human prostate cancer cells (Months 24-36):

We have not addressed this task partly or fully as yet.

Key Research Accomplishments:

1. PC3 human prostate cancer cells infected with Tbx2 dominant negative construct form lesions in the tibia of nude mice that are smaller and less osteolytic as compared to controls.

2. Bigenic mice over-expressing both hepsin and myc in the mouse prostate develop adenocarcinoma at a faster rate as compared to myc alone mice. The hepsin/myc bigenic mice of higher ages develop a higher grade adenocarcinoma as compared to age matched myc alone mice.

3. The expression of hepsin in the hepsin/myc transgenic mice resulted in the degradation of Laminin-332, a basement membrane component that is lost during the progression of human prostate cancer.

Reportable Outcomes:

1. Development of tibial grafts using PC3-EV and PC3-Tbx2 DN vector.

2. Development of a bigenic mouse model of prostate cancer over-expressing hepsin and myc.

3. Presented a poster at the Society of Basic Urologic Research (SBUR) Annual Fall Meeting Nov 2008, Phoenix, AZ. titled "Tbx2 mediates osteogenic burden of PC3 human prostate cancer cells in bone microenvironment."

4. Co-author on a journal paper (Ref 1). This study was performed in collaboration with our lab.

Conclusions:

Blocking endogenous TBX2 levels in PC3 human prostate cancer cells by utilizing a dominant negative construct and injecting the cells in mouse tibia lead to lesions that had reduced osteolytic burden as compared to control PC3 cells. We are currently quantifying these bone lesions by micro CT scanning. In an effort to create a prostate cancer bone metastasis model and study the role of Tbx2 in bone metastasis, we have created a bigenic mouse over-expressing hepsin and myc in the mouse prostate. Though these bigenic mice do not develop any metastases, these mice develop invasive adenocarcinoma at a faster rate as compared with myc

mice alone. Further, the hepsin/myc mice of higher ages display a pathologically higher grade of adenocarcinoma as compared with age matched myc mice.

References:

1. Tripathi, M., Nandana, S., Yamashita, H., Ganesan, R., Kirchhofer, D., and Quaranta, V. Laminin-332 is a substrate for hepsin, a protease associated with prostate cancer progression, J Biol Chem, 283 : 30576-30584, 2008.

Appendices:

N/A

Supporting Data:

Fig. 1: Schematic of intra-tibial injection

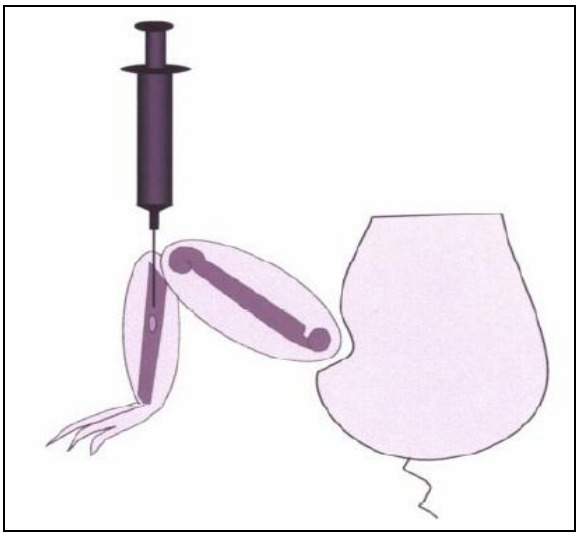

Fig. 2: PC-Tbx2 DN cells decrease osteolytic burden when injected into the tibia of nude mice.

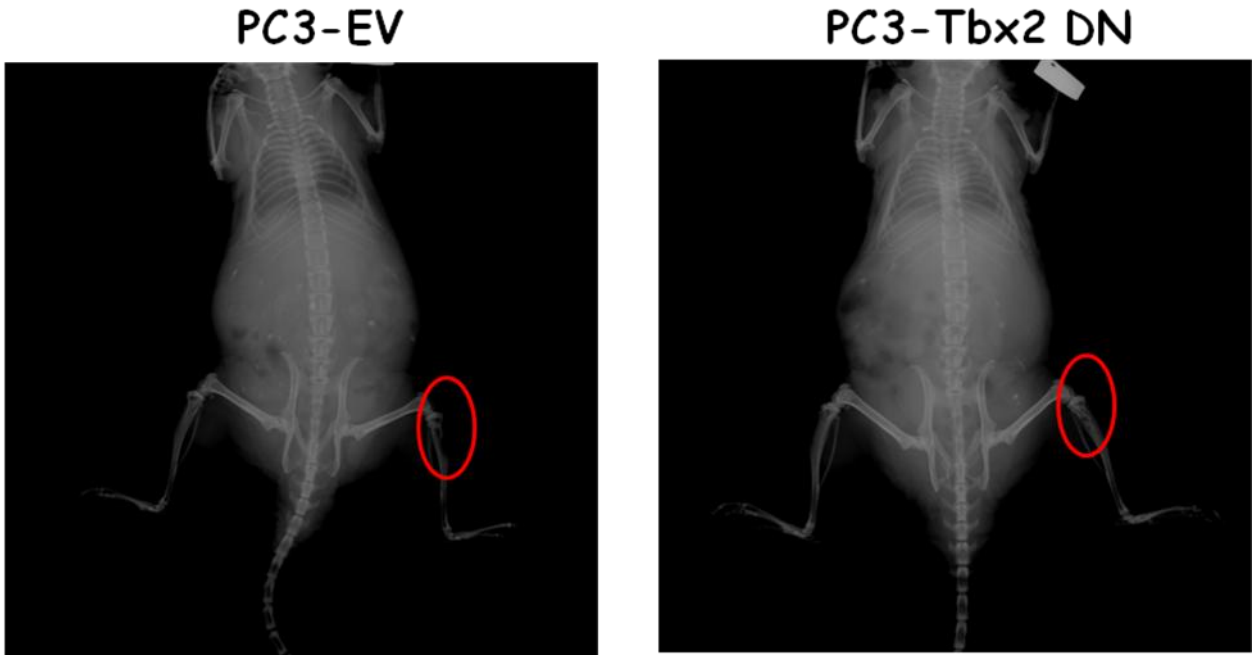

Fig 3: H&E staining of tibia injected with PC3-EV and PC3-Tbx2 DN respectively.